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Characterization of thermophilic fungal community associated with pile fermentation of Pu-erh tea

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ABSTRACT

This study aimed to characterize the thermophilic fungi in pile-fermentation process of Pu-erh tea. Physicochemical analyses showed that the high temperature and low pH provided optimal conditions for propagation of fungi. A number of fungi, including *Blastobotrys adeninivorans*, *Thermomyces lanuginosus*, *Rasamsonia emersonii*, *Aspergillus fumigatus*, *Rhizomucor pusillus*, *Rasamsonia byssochlamydoides*, *Rasamsonia cylindrospora*, *Aspergillus tubingensis*, *Aspergillus niger*, *Candida tropicalis* and *Fusarium graminearum* were isolated as thermophilic fungi under combination of high temperature and acid culture conditions from Pu-erh tea pile-fermentation. The fungal communities were analyzed by PCR-DGGE. Results revealed that those fungi are closely related to *Debaryomyces hansenii*, *Cladosporium cladosporioides*, *A. tubingensis*, *R. emersonii*, *R. pusillus*, *A. fumigatus* and *A. niger*, and the last four presented as dominant species in the pile process. These four preponderant thermophilic fungi reached the order of magnitude of 10^7, 10^7, 10^7 and 10^6 copies/g dry tea, respectively, measured by real-time quantitative PCR (q-PCR). The results indicate that the thermophilic fungi play an important role in Pu-erh tea pile fermentation.

1. Introduction

Pu-erh tea is a unique full-fermented tea, which is primarily manufactured in Yunnan province, China. It possesses reddish to brownish red or with gray appearance, thick and bright red infusion color, bittersweet taste and a unique moldy odor which becomes more prominent as the fermentation continues and the leaves age (Zhou et al., 2004). Pu-erh tea has been traded on the Tea Horse Road from Yunnan to the Tibetan Plateau and Southeast regions of Asia for thousands of years (Yang, 2009). Today, in addition to being a favorite drink for residents in China and Southeast Asia, it has also been exported to Japan, USA, Britain and other countries. Recent studies have demonstrated multifaceted benefits of Pu-erh tea as a functional beverage including anti-oxidative (Duh et al., 2010; Fan et al., 2013), anti-mutagenic (Wu et al., 2007), antibacterial (Wu et al., 2007; Hu et al., 2010), antiviral (Pei et al., 2011; Huang et al., 2012), anti-tumor (Zhao et al., 2011), cholesterol-lowering (Peng et al., 2013), anti-obesity (Oi et al., 2012), hypoglycemic (Du et al., 2012) and anti-allergic (Yamazaki et al., 2012) activities.

The raw material of Pu-erh tea is made from the leaves and buds of a broad-leaf variety of the tea plant (*Camellia sinensis var. assamica* (L.) O. Kuntze; Theaceae) by heating, rolling and drying, similar to the manufacture of green tea. In the past, large batches of Pu-erh tea were transported for months on horseback to Tibet and other remote destinations. The tea matured continuously along the way. From the 1970s, most Pu-erh tea goes through a high temperature pile-fermentation in order to hasten aging. It is reported that very complex changes take place to form its unique black tea quality due to microbial thermogenesis combined with microbial metabolism, natural oxidation and extracellular enzyme activities (Xu et al., 2005; Ahmed and Stepp, 2013; Lv et al., 2013). It is believed that fungi play a decisive role in the formation of the tea characteristics such as the unique musty flavor in the production process (Zhao et al., 2013a).

In order to reveal the complex fungal communities and their impact on the quality of Pu-erh tea, some researchers investigated commercial Pu-erh tea products. Zhao et al. (2010) investigated 60 samples of Pu-erh tea and concluded that the predominant fungi were yeast and different species of genus *Aspergillus*, followed by *Penicillium*. In addition, Haas et al. (2013) reported fungi such as *Aspergillus*, *Penicillium*, *Rhizomucor*, *Mucor*, *Cladosporium* and *Eurotium* species from 36 Pu-erh tea samples. *A. niger* was believed to be the dominant fungus contributing to the quality of production (Abe et al., 2008; Chen et al., 2009). However, unlike room temperature and low moisture content of the commercial product, Pu-erh tea pile-fermentation is a high temperature and high moisture production process. It may have significantly

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different thermophilic fungal communities compared with commercial products. Publications of microorganism profiles during the pile-fermentation process are limited. Xu et al. (2005) and Abe et al. (2008) isolated *A. niger* and *Blastobotrys adeninivorans* from pile-fermentation Pu-erh tea samples incubated at 25 and 30 °C respectively.

In 1993, Muyzer et al. (1993) were the first to use polymerase chain reaction —denaturing gradient gel electrophoresis (PCR-DGGE) method to analyze complex microbial communities. Since then this culture-independent technique has been widely applied to the study of microbial communities of fermenting food, including tea. Abe et al. (2008) confirmed the existence of *A. niger* and *B. adeninivorans* detected by PCR-DGGE. Tian et al. (2013) also used PCR-DGGE to with a culture-dependent method to draw the same conclusion that yeast, *Aspergillus* sp. and *Penicillium* sp. were the dominant fungi in different aged Pu-erh tea.

A comprehensive research of fungal communities during the fermentation may help clarify the mechanism of how the sensory quality of Pu-erh tea develops. In this work, the whole fermentation process of Pu-erh tea has been studied. Thermophilic fungi emerging in the process were analyzed by both culture-dependent dilution plating of high temperature and culture-independent PCR-DGGE and real-time quantitative PCR (q-PCR) to determine their diversity and dynamics.

2. Materials and methods

2.1. Manufacture process of Pu-erh tea

The batch of Pu-erh tea fermentation for this study was manufactured in the Demonstration Plant of Pu'er Tea, Institute of Pu-erh Tea, Pu-Er city of Yunnan province from September 15 to November 2, 2012. A total of five tons tea leaves harvested from the Tea Seed Manipulation Farm, Pu'er city, were used in this pile-fermentation process. Tea leaves were first passed transiently through a semi-automatic rotary kiln to remove moisture and inactivate the enzymes in the fresh leaves. Then the leaves were rolled to soften leaves and buds. Rolled leaves were then spread and dried in the sunlight to obtain the raw material. Blends of the materials were first wet with water to reach an average water content of 45% and piled to about one meter height to start the fermentation. During the fermentation, periodic turning over was carried out on Sep. 23, Sep. 30, Oct. 11, Oct. 20 and Oct. 29 in order to improve homogeneity and avoid overheating. At the end of the fermentation, a kind of dry method called “Tonggou” was used to reduce the water content of the tea to avoid over-fermenting. All processes were performed in accordance with traditional method by skilled workers.

2.2. Sampling and physicochemical characterization

Pile-fermentation was carried out for approximately 49 days, and aseptic sampling was performed at 8:00 am each day from five representative points of the bulk. Sampling points were distanced from the edge of the bulk one meter or more, and were not repeated at the same place. The obtained samples (total about 1.0–1.5 kg wet) were mixed evenly and packaged in sterile self-sealing bags. The temperature, humidity of the workshop and pile temperature were recorded each day at 8:00, 11:30 and 17:30. The water content of the leaf samples (about 100 g) was calculated after drying at 103 °C for 5 h. Tea sample of 1 g was added to 5 mL of distilled water, stirred with a glass rod and pH was measured with a precision pH meter.

2.3. Isolation and identification of fungi

YPD agar (10 g yeast extract, 20 g peptone, 20 g glucose, 15 g agar, L^{-1}; pH 5.0) was used as basic culture medium. Fermenting tea leaves (10 g) were suspended in 90 mL of sterile water and homogenized in a bagmixer for 3 min. Then 10^3, 10^4, and 10^5-fold dilutions of the homogenized suspension were spread onto agar plates and incubated at 50, 55 or 60 °C, respectively.

The purified fungi isolates were identified by sequencing analysis of the ITS region fragment of rRNA gene. The primers used for the amplification were ITS1F and ITS4 (Table S1), and PCR amplification was performed using KOD Fx (TOYOBO, Osaka, Japan). Fungus PCR system and condition for the amplification were the same as those used for PCR-DGGE analysis. The DNA sequencing was accomplished by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd., China.

2.4. DNA extraction of tea samples

A tea sample of 10 g was put into a sterile bag, 90 mL sterilized 0.8% Tween buffer added, then beaten for 3 min to obtain the microorganism suspension. A1 mL aliquot of the suspension was transferred into a 2 mL microcentrifuge tube, briefly centrifuged at 12,000 rpm for 2 min to recover the microbes as a pellet. DNA extraction from the pellet was conducted using Dneasy plant Mini Kit (QIAGEN GmbH, Germany) according to the manufacturer's instruction with minor modifications in speed and time for centrifugation (which were slightly increased).

2.5. PCR amplification of ITS region for DGGE analysis

The extracted DNA was subject to amplification of the ITS region. The primers used in this study for DGGE analysis are ITS1F-GC and ITS4 (Table S1), synthesized by Sunbiotech Co., Ltd. (Beijing, China). The PCR reactions were performed using a MyCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Amplifications were carried out in a final volume of 50 μL, containing 2.5 μL of template, 25 μL of the 2× PCR buffer, 10 μL of dNTP mixture (2 mM), 1 μL of KOD Fx (TOYOBO, Osaka, Japan) (1.0 U/μL), 0.75 μL each primer (20 μmol/L) and 10 μL of sterilized distilled water. All PCR reactions were as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and extension at 68 °C for 2 min. DGGE analysis of the amplification products was equipped with a DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). PCR products (30 μl each) were supplied in polyacrylamide gel (8% w/v acrylamide-bisacrylamide at 37.5:1) using a denaturing gradient from 30% to 60% urea-formamide (100% corresponding to 7 M urea and 40% w/v deliomized formamide), increasing in the direction of the electrophoretic run. The electrophoretic run was carried out at a constant temperature of 60 °C in 1.0× Tris-acetate-EDTA (TAE) at 180 V for 5.5 h.

2.6. Quantification of dominant species by q-PCR

Aspergillus fumigatus, *Rhizomucor pusillus*, *A. niger*, and *Rasamsonia emersonii* were selected for quantification by real-time quantitative PCR. The primers of each species for q-PCR assay were designed and listed in Table S1. Amplification and detection of DNA by q-PCR were performed with the ABI 7500 Real-Time PCR Systems (Applied Biosystems). The PCR reaction was performed in a total volume of 20 μL using the UltraSYBR Mixture with Rox (CWbio. Co. Ltd. Beijing, China), containing 10 μL of 2 × UltraSYBR Mixture, 0.4 μL of each of the primers (10 μM), 2 μL of template DNA and H_2O. UltraSYBR was used as a fluorescent dye, which could quantify thermal denaturation accompanying software to perform melting curves (Gonzalez and Saiz-Jimenez, 2005; Huang et al., 2014). Gene copy numbers were determined according to a standard curve as described previously (Ye et al., 2011; Jamal et al., 2012). The standard curve was obtained by gradient plasmid dilutions of a quantitative plasmid DNA.